Selective Replenishment of Two Vesicle Pools Depends on the Source of Ca^{2+} at the *Drosophila* Synapse

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Summary

After synaptic vesicles (SVs) undergo exocytosis, SV pools are replenished by recycling SVs at nerve terminals. At *Drosophila* neuromuscular synapses, there are two distinct SV pools (i.e., the exo/endo cycling pool (ECP), which primarily maintains synaptic transmission, and the reserve pool (RP), which participates in synaptic transmission only during tetanic stimulation). Labeling endocytosed vesicular structures with a fluorescent styryl dye, FM1-43, and measuring intracellular Ca^{2+} concentrations with a Ca^{2+} indicator, rhod-2, we show here that the ECP is replenished by SVs endocytosed during stimulation, and this process depends on external Ca^{2+}. In contrast, the RP is refilled after cessation of tetanus by a process mediated by Ca^{2+} released from internal stores.

Introduction

Upon nerve stimulation, synaptic vesicles (SVs) undergo exocytosis at the nerve terminal, and the pool of SVs is replenished by endocytosis (Heuser and Reese, 1973; Ceccarelli et al., 1973). There are functionally and morphologically distinct pools of SVs in the nerve terminal (Birks and MacIntosh, 1961; Pieribone et al., 1995; Kuromi and Kidokoro, 1998; Schikorski and Stevens, 2001). One SV pool is small and readily releasable, while the other is larger and serves as a reservoir to supply SVs during high-frequency firing of nerves (Pieribone et al., 1995; Kuromi and Kidokoro, 1998). The time course of replenishment of the readily releasable pool has been studied electrophysiologically (Dittman and Regehr, 1998; Wang and Kaczmarek, 1998). However, it is unknown whether the replenishment is achieved by recruitment of SVs from other pools or by endocytic recycling. When endocytosis is blocked at nonpermissive temperatures in a temperature-sensitive paralytic *Drosophila* mutant, *shibire*, nerve terminals are completely depleted of SVs within a short period of time (Poodry and Edgar, 1979; Koenig et al., 1983). Thus, recycling of SVs from the plasma membrane is an essential mechanism for replenishment of SV pools. To study the SV recycling process, Betz and Bewick (1992) developed a method to label endocytosed SVs with a fluorescent styryl dye, FM1-43. The dye is taken up into SVs by endocytosis and released by exocytosis. Using this technique, we have demonstrated two SV pools in presynaptic boutons at *Drosophila* neuromuscular synapses (i.e., ECP and RP) (Kuromi and Kidokoro, 1998). It remains unknown, however, how each SV pool is refilled by endocytosis.

It has been suggested that Ca^{2+} plays significant roles in recycling of SVs in the nerve terminal and in replenishment of the readily releasable pool (Ceccarelli et al., 1973; Dittman and Regehr, 1998; Sankaranarayanan and Ryan, 2001). An elevation of the external Ca^{2+} concentration increased the number of docked vesicles per release site at the *Drosophila* synapse (Koenig et al., 1993). At the mouse auditory relay synapse, the replenishment of releasable pool after stimulation was enhanced by high-frequency stimulation but reduced by EGTA or Cd^{2+}, suggesting that Ca^{2+} entry through voltage-gated Ca^{2+} channels enhances replenishment (Wang and Kaczmarek, 1998).

In this study, we examined experimental conditions in which the two SV pools at *Drosophila* neuromuscular synapses are selectively refilled by endocytosis and the role of Ca^{2+} for replenishment. We found that the ECP is replenished by SVs endocytosed during stimulation, and this process requires external Ca^{2+}. In contrast, the RP is refilled after cessation of tetanus by a process mediated by Ca^{2+} released from internal stores.

Results

We labeled synaptic vesicles (SVs) in presynaptic boutons at the neuromuscular synapse of *Drosophila* larvae with FM1-43. In this article, we refer to the SV pool, which is located at the periphery of boutons and released by high K^+ stimulation as is the exo/endo cycling pool (ECP), and to the pool, which is located broadly toward the center of boutons and not released by high K^+ stimulation, as is the reserve pool (RP) (Kuromi and Kidokoro, 1998, 2000).

Replenishment of SV Pools Depends on Stimulus Frequency and Timing of Endocytosis Relative to Stimulation

To determine the condition in which each SV pool is refilled by endocytosed SVs, the loading process of FM1-43 into individual boutons was varied by presenting the dye during ("simultaneous load") or after ("delayed load") stimulation at a low (3 Hz) or a high (30 Hz) frequency. When the ventral nerve cord was stimulated at 3 Hz in normal saline (2 mM Ca^{2+}) containing FM1-43, the periphery of boutons was stained (Figure 1A, top; the fluorescence intensity is indicated by the height of column in Figure 1E labeled simultaneous load) and subsequently destained with 90 mM K^+ treatment for 5 min in the absence of the dye (high K^+ unloading) (Figure 1A, bottom; the intensity after high K^+ unloading is indicated by the height of stippled column in Figure 1E). When the ventral nerve cord was stimulated at 3 Hz in normal saline without the dye and then exposed to normal saline containing FM1-43 without stimulation, no fluorescence was observed in boutons (Figure 1B; column in Figure 1E labeled delayed load is not discernible from the base line).

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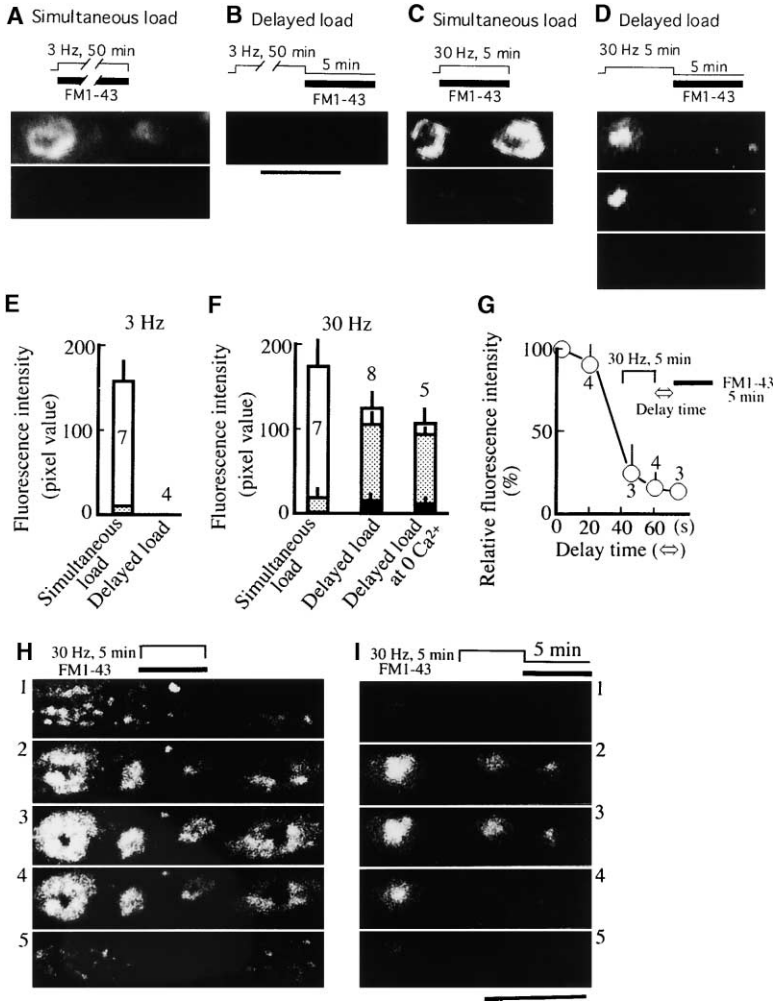

Figure 1. FM1-43 Loading into Presynaptic Boutons during and after Stimulation at 3 Hz or 30 Hz in Normal Saline

Stimulation and FM1-43 loading protocols are shown at the top of (A)–(D), (H), and (I).

(A) The preparation was stimulated at 3 Hz for 50 min in normal saline with 10 μM FM1-43 and washed with Ca^{2+}-free saline for 10 min (top). Subsequently, it was treated with 90 mM K^+ saline containing 2 mM Ca^{2+} for 5 min in the absence of dye and then washed with Ca^{2+}-free saline (high K^+ unloading) (bottom).

(B) The preparation was stimulated at 3 Hz for 50 min in normal saline without dye, then incubated for 5 min in normal saline containing 10 μM FM1-43 without stimulation and washed. No FM1-43 was taken up in boutons. Boutons were identified by Nomarski optics.

(C) 10 μM FM1-43 was present in normal saline during stimulation at 30 Hz for 5 min and then washed (top). The dye was completely released after high K^+ unloading (bottom).

(D) The preparation was stimulated at 30 Hz for 5 min in normal saline without dye, then incubated for 5 min in normal saline containing 10 μM FM1-43 without stimulation and washed (top). Some dye remained after high K^+ unloading (middle). The preparation was subsequently stimulated at 30 Hz for 5 min in normal saline without dye (bottom).

(E and F) The FM1-43 fluorescence intensity (mean pixel value) in boutons loaded with a protocol of either simultaneous or delayed loads was measured before (height of whole columns) and after high K^+ unloading (height of stippled columns), and after subsequent stimulation at 30 Hz for 5 min (height of filled columns). The preparation was incubated for 5 min in Ca^{2+}-free saline containing 10 μM FM1-43 after stimulation for 5 min at 30 Hz in normal saline without dye (delayed load at 0 Ca^{2+}). The number of preparations examined is indicated at each column. Vertical bars attached to each column are SEM.

(G) The time course of FM1-43 uptake after cessation of tetanus. The relative fluorescence intensity (Ft/F0 × 100) is plotted against delay time (t) between cessation of stimulus and FM1-43 application. Ft is the fluorescence intensity at time t, and F0 is that at time 0.

(H and I) Two series of confocal views of boutons stained with FM1-43 by simultaneous (H) and delayed (I) loads. The confocal plane was stepped by 0.5 μm between successive images, including the brightest optical section. In (H), FM1-43 was present during stimulation at 30 Hz for 5 min in normal saline. Relative distance: 1; −1.5, 2; −1.0, 3; 0.0, 4; +1.0, 5; +2.5 μm In (I), FM1-43 was applied for 5 min in normal saline after stimulation at 30 Hz for 5 min. Relative distance: 1; -0.5, 2; 0.0, 3; +0.5, 4; +1.0, 5; +1.5 μm.

Scale bar: 5 μm in (A)–(D), (H), and (I).

When the nerve was stimulated at 30 Hz in normal saline containing FM1-43, the periphery of boutons was also predominantly stained (Figure 1C, top; column in Figure 1F labeled simultaneous load), and destained by high K^+ unloading (Figure 1C, bottom; stippled column in Figure 1F labeled simultaneous load). In contrast, when FM1-43 was applied after cessation of tetanus, FM1-43 fluorescence was found in the center of boutons (Figure 1D, top; column in Figure 1F labeled delayed load). In this case, most of fluorescence remained even after high K^+ unloading (Figure 1D, middle; stippled column in Figure 1F labeled delayed load). The remainer of the fluorescence virtually disappeared after tetanus without the dye (Figure 1D, bottom; filled column in Figure 1F labeled delayed load), as reported previously (Kuromi and Kidokoro, 2000; Delgado et al., 2000).

We then determined the dependence of endocytosis on delay time after tetanus (Figure 1G). After a delay time following the end of tetanus, the preparation was exposed to FM1-43 in normal saline. The amount of FM1-43 loaded in the center decreased as the dye presentation after tetanus was delayed, in accordance with the previous results (Ryan et al., 1996; Wu and Betz, 1996).

To confirm the subcellular localization of ECP and RP, we examined the distribution of FM1-43 within single boutons three-dimensionally with a confocal microscope. SVs endocytosed during and after tetanus were found to localize predominantly at the periphery and center of boutons, respectively (Figures 1H and 1I).

The above results demonstrate that in normal saline, the ECP is replenished during simultaneous load and that the RP is refilled during delayed load after tetanus and unloaded by tetanic stimulation.

Roles of External Ca^{2+} in Replenishment of SV Pools during and after Tetanus and in Exocytosis

Figure 2A compares the staining patterns of identified boutons loaded with FM1-43 at two different external Ca^{2+} concentrations during tetanus. The periphery of boutons was stained at 2 mM Ca^{2+} and completely destained by high K^+ unloading (Figure 2A, rows 1 and 2). In contrast, at 0.2 mM Ca^{2+}, the FM1-43 fluorescence was observed predominantly in the center, and the fluorescence remained after high K^+ unloading (Figure 2A, rows 3 and 4). Thus, at low, external Ca^{2+}, the endocytosed SVs were incorporated into the RP even by simultaneous load. Figure 2B shows a fluorescence image of boutons that were exposed to FM1-43 in Ca^{2+}-free saline after the nerve had been tetanically stimulated in normal saline. The center of boutons was primarily stained, and the fluorescence remained after high K^+ unloading (Figures 2B1 and 2B2; column in Figure 1F labeled delayed load at 0 Ca^{2+}). This staining pattern was the same as that observed in normal saline (Figure 1D; column in Figure 1F labeled delayed load). These results suggest that the endocytic replenishment of RP after tetanus was not affected by external Ca^{2+}, while incorporation of SVs endocytosed during tetanus into pools is qualitatively changed by external Ca^{2+} concentrations.

We further examined the relationship between the concentration of external Ca^{2+} and FM1-43 uptake into SV pools during tetanus. Since endocytosis is most likely to be coupled to exocytosis, we also investigated the effect of Ca^{2+} concentration on exocytosis. Exocytosis during tetanus diminished as the Ca^{2+} concentration decreased and was abolished at 0 mM Ca^{2+} (Figure 2C, right, filled columns). With a decrease in the Ca^{2+} concentration, FM1-43 uptake into the ECP during tetanus also declined (blank portions of left columns in Figure 2C). The differences in the height of blank portions of left columns were significant, between 2 and 1 mM Ca^{2+} and between 1 and 0.2 mM ($p < 0.01$). In contrast, FM1-43 uptake into the RP at 2 and 20 mM Ca^{2+} was significantly lower than that at 0.2 and 1 mM Ca^{2+} ($p < 0.05$, left, stippled columns in Figure 2C). Thus, the replenishment of ECP by SVs endocytosed during tetanus depends on the external Ca^{2+} concentration, while that of the RP occurs with lower Ca^{2+} concentrations and declines with higher concentrations.

Thapsigargin Enhanced Elevation of $[Ca^{2+}]_i$ in Early Phase of Tetanus but Prevented Persistence of Elevated $[Ca^{2+}]_i$ after Tetanus

Two major intracellular organelles, endoplasmic reticulum (ER) and mitochondria, constitute internal Ca^{2+} stores in the nerve terminal. To determine the role of internal Ca^{2+} stores in the replenishment, we manipulated internal Ca^{2+} stores with pharmacological means. Thapsigargin is known to inhibit Ca^{2+} uptake into the ER. Its application results in an initial increase in the cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_i$, and subsequent depletion of Ca^{2+} in the ER (Thastrup et al., 1990). Changes in $[Ca^{2+}]_i$ in boutons were monitored in untreated and thapsigargin-treated (20 μM for 20 min in normal saline or in Ca^{2+}-free saline) preparations by loading boutons with a Ca^{2+} indicator, rhod-2/AM (Melamed et al., 1993; Kamiya and Ozawa, 2000). In controls,

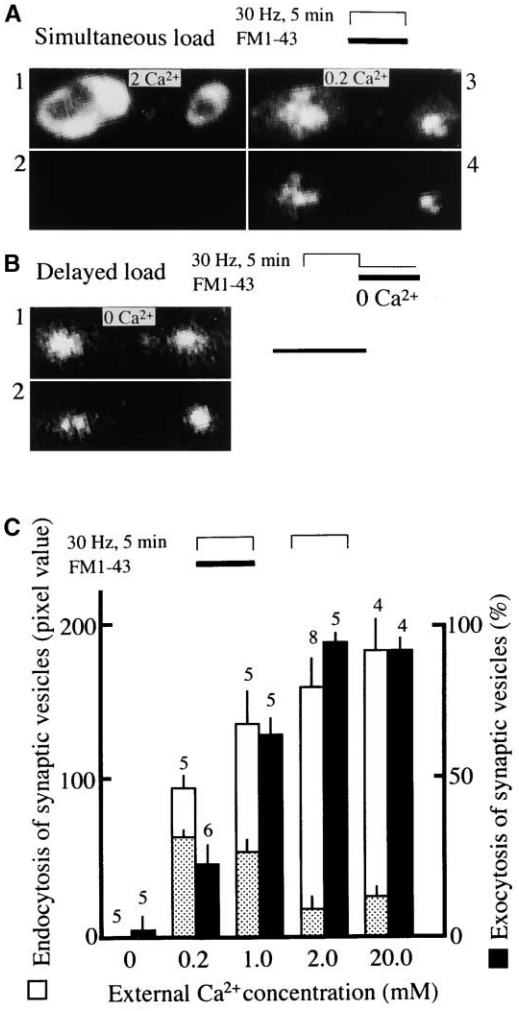

Figure 2. Effects of External Ca^{2+} on FM1-43 Loading into the SV Pools during and after Tetanus and on Exocytosis of SVs

(A) Simultaneous load at 2 mM (rows 1 and 2) and 0.2 mM Ca^{2+} (rows 3 and 4). The nerve was stimulated at 30 Hz for 5 min in normal saline (2 mM Ca^{2+}) containing 10 μM FM1-43 and washed (row 1). Rows 2 and 4 show effects after high K^+ unloading. The same boutons were then stimulated at 30 Hz for 5 min in the medium with 0.2 mM Ca^{2+} containing 10 μM FM1-43 and washed (row 3).

(B) Delayed load at 0 Ca^{2+}. The nerve was stimulated at 30 Hz for 5 min in normal saline without dye, then incubated in Ca^{2+}-free saline containing 10 μM FM1-43 without stimulation and washed (row 1). Row 2 shows after high K^+ unloading.

(C) Exocytosis and endocytosis of SVs during stimulation at 30 Hz for 5 min at various external Ca^{2+} concentrations. To measure exocytosis of SVs, boutons were preloaded with FM1-43 by incubating for 5 min in 90 mM K^+ saline (2 mM Ca^{2+}) containing 10 μM FM1-43 and washed. The FM1-43 fluorescence intensity in boutons was measured before and after stimulation at 30 Hz for 5 min at various concentrations of Ca^{2+} without dye. The extent of exocytosis was expressed as the percentage of FM1-43 fluorescence released from boutons during stimulation to the fluorescence intensity before stimulation (filled columns). The extent of endocytosis of SVs was determined by measuring FM1-43 taken up into boutons during stimulation at 30 Hz for 5 min at various concentrations of Ca^{2+}. The FM1-43 fluorescence intensity in boutons before (height of whole columns at left) and after high K^+ unloading (height of stippled columns).

Scale bar: 5 μm in (A) and (B).

the rhod-2 fluorescence intensity in boutons increased during stimulation at 30 Hz and declined gradually after tetanus but stayed at a slightly elevated level for a prolonged time (Figures 3A; open circles in Figure 3C labeled Ctrl). In contrast, in preparations treated with thapsigargin in normal saline, the rhod-2 fluorescence intensity increased to a higher peak during early phase and declined during tetanus. After tetanus, the fluorescence rapidly declined to the pretetanus level (Figure 3B; closed circles in Figure 3C labeled Thaps at 2 Ca^{2+}). When preparations were treated with thapsigargin in Ca^{2+}-free saline and stimulated in normal saline, the higher peak of rhod-2 fluorescence during early phase of tetanus was not observed, and the residual elevation in fluorescence after tetanus was abolished (Figure 3C, closed triangles labeled Thaps at 0 Ca^{2+}). These changes in rhod-2 fluorescence in boutons are consistent with the notion that thapsigargin depletes internal Ca^{2+} stores by inhibiting Ca^{2+} uptake into the ER (Thastrup et al., 1990) and suggest that the prolonged elevation of $[Ca^{2+}]_i$ in boutons after tetanus is maintained by Ca^{2+} released from internal stores.

Roles of Internal Ca^{2+} Stores in Replenishment of SV Pools during and after Tetanus and in Exocytosis

Having examined the changes of $[Ca^{2+}]_i$ in boutons in thapsigargin-treated preparations, we next investigated the replenishment of SV pools. When thapsigargin-treated boutons were loaded with FM1-43 by delayed load, no FM1-43 fluorescence was observed in boutons (Figure 4C; column in Figure 4F labeled Thaps), while in untreated preparations, FM1-43 was predominantly loaded into the center, and the fluorescence remained after high K^+ unloading (Figure 4F, column labeled Ctrl). Thus, thapsigargin inhibited the replenishment of RP after tetanus, corresponding to no residual elevation of $[Ca^{2+}]_i$ in boutons after tetanus in thapsigargin-treated preparations (Figure 3B, far right; Figure 3C, closed circles and triangles).

When FM1-43 was loaded into boutons during stimulation at 30 Hz for 2 min in thapsigargin-treated preparations (20 μM for 20 min in normal saline), whole boutons were stained, and fluorescence in the center remained after high K^+ unloading (Figure 4A, top and bottom; column in Figure 4D labeled Thaps). This is in contrast to untreated controls in which only the periphery was stained and the fluorescence disappeared after high K^+ unloading (Figure 4D, column labeled Ctrl). Exocytosis of SVs was also enhanced in thapsigargin-treated preparations (Figure 4G, column labeled Thaps). This result probably corresponds to a higher level of $[Ca^{2+}]_i$ in boutons in the treated preparations (Figure 3B; closed circles in Figure 3C, during the early phase of tetanus). However, when FM1-43 was applied to thapsigargin-treated preparations during the late phase of tetanus between 3 and 5 min, only the periphery of boutons was stained and destained by high K^+ unloading (Figure 4B, top and bottom; column in Figure 4E labeled Thaps). This staining pattern is the same as that observed in untreated preparations (Figure 4E, column labeled Ctrl). These results suggest that the incorporation of FM1-43 into the RP during the early phase of tetanus in thapsi-

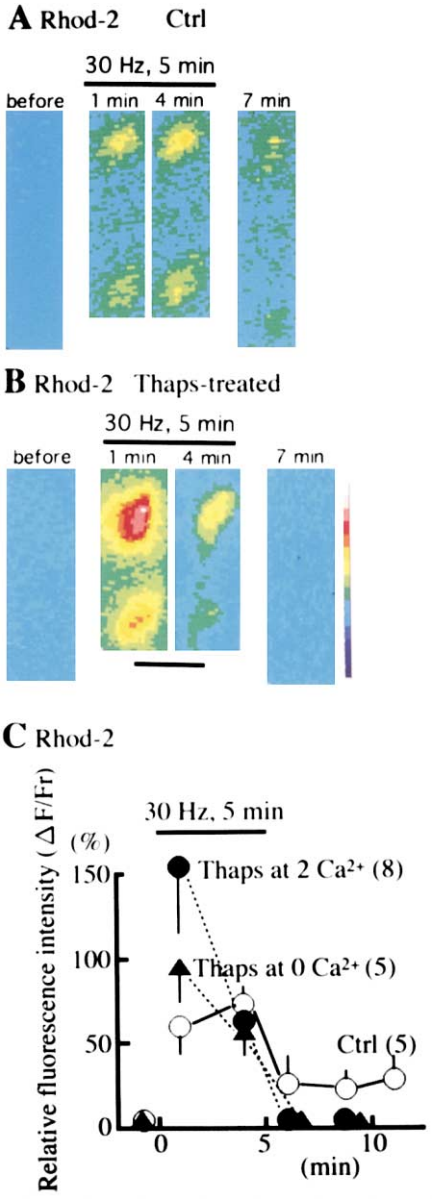

Figure 3. Changes in $[Ca^{2+}]_i$ in Boutons during and after Tetanus in Control and Thapsigargin-Treated Preparations

(A and B) Pseudo-colored rhod-2 images of boutons in untreated (A) and thapsigargin-treated (20 μM for 20 min in normal saline) (B) preparations. To measure $[Ca^{2+}]_i$ in boutons, preparations were incubated for 40 min in nominally 0 Ca^{2+} saline containing 10 μM rhod-2/AM and 0.03% pluronic F-127. The nerve was stimulated at 30 Hz for 5 min in normal saline. Warmer colors correspond to higher $[Ca^{2+}]_i$.

(C) Changes of $[Ca^{2+}]_i$ in thapsigargin-treated in normal saline (closed circles labeled as Thaps at 2 Ca^{2+}), thapsigargin-treated in Ca^{2+}-free saline (closed triangles labeled as Thaps at 0 Ca^{2+}), and untreated (open circles labeled as Ctrl) boutons during and after tetanus. The resting fluorescence (Fr) was subtracted from the stimulation-induced value (Ft), and the result ($\Delta F = Ft - Fr$) was normalized to the resting fluorescence intensity (Fr) to give $\Delta F/Fr \times 100$ and plotted against time after the onset of tetanus. Changes in fluorescence intensity in two to three boutons were averaged in one preparation. Numbers in the parentheses is the number of preparations examined. Vertical bars attached each symbol are SEM.

Scale bar: 5 μm in (A) and (B).

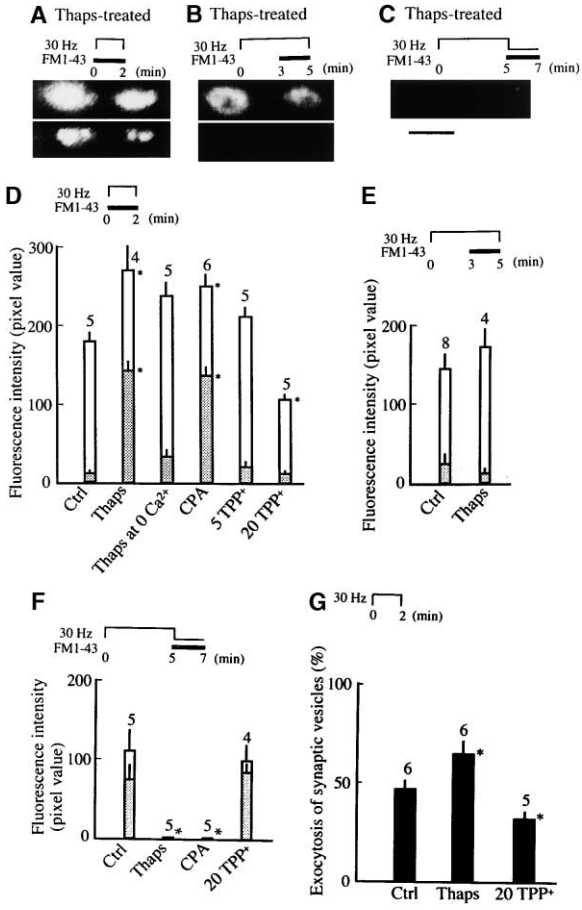

Figure 4. Effects of Internal Ca^{2+} Stores on FM1-43 Loading into SV Pools during and after Tetanus and on Exocytosis of SVs

(A–C) Thapsigargin (20 μM) was applied in normal saline starting at 20 min before stimulation through the period of FM1-43 loading. In (A), 10 μM FM1-43 was applied for 2 min during stimulation at 30 Hz (top) and after high K^+ unloading (bottom). In (B), the nerve was stimulated at 30 Hz for 5 min. 10 μM FM1-43 was applied between 3 and 5 min during the period of stimulation (top) and after high K^+ unloading (bottom). In (C), the nerve was stimulated at 30 Hz for 5 min and 10 μM FM1-43 was applied for 2 min without stimulation.

(D) FM1-43 was loaded into boutons by applying in the period between 0 and 2 min during stimulation at 30 Hz. The FM1-43 fluorescence intensity in boutons before (height of whole columns) and after high K^+ unloading (height of stippled columns). Thaps, CPA, 5 TPP^+, and 20 TPP^+ indicate preparations which were treated with 20 μM thapsigargin, 10 μM CPA, 5 μM TPP^+, and 20 μM TPP^+ in normal saline, respectively, and loaded with FM1-43. Thaps at 0 Ca^{2+} indicates preparations which were treated with 20 μM thapsigargin in Ca^{2+}-free saline for 20 min and loaded with FM1-43 in normal saline. Ctrl indicates untreated preparations.

(E) FM1-43 was loaded into boutons by applying in the period between 3 and 5 min during stimulation at 30 Hz. Thaps indicates thapsigargin-treated (in normal saline), and Ctrl indicates untreated preparations.

(F) FM1-43 was loaded into boutons by applying for 2 min after stimulation at 30 Hz for 5 min (delayed load). Ctrl, Thaps, CPA, and 20 TPP^+ indicate untreated, thapsigargin-treated (in normal saline), CPA-treated, and TPP^+-treated (20 μM) preparations, respectively.

(G) Effects of thapsigargin and TPP^+ on exocytosis of SVs. The extent of exocytosis was measured and expressed as described in the figure legend to Figure 2C. Ctrl, Thaps, and 20 TPP^+ columns indicate untreated, thapsigargin-treated (normal saline), and TPP^+-treated (20 μM) preparations, respectively. Significant difference is $p < 0.01$ compared with control values in (D)–(G).

Scale bar: 5 μm in (A)–(C).

gargin-treated preparations is induced by a higher level of $[Ca^{2+}]_i$ in boutons due to Ca^{2+} release from internal stores and inhibition of Ca^{2+} sequestration into the ER. The following results support this interpretation. To deplete internal Ca^{2+} stores, we treated preparations with thapsigargin in Ca^{2+}-free saline (Figure 3C, triangles). We then loaded the preparations with FM1-43 during stimulation at 30 Hz for 2 min in normal saline. The periphery of boutons was predominantly stained with FM1-43, and the remaining fluorescence after high K^+ unloading was significantly decreased compared with that in thapsigargin-treated preparations in normal saline (Figure 4D, columns labeled Thaps at 0 Ca^{2+} and Thaps).

Cyclopiazonic acid (CPA; 50 μM for 10 min in normal saline), another depleting agent of ER Ca^{2+} stores, had similar effects as thapsigargin on simultaneous and delayed loading of FM1-43 into boutons (Figures 4D and 4F, columns labeled CPA). These results are consistent with those of thapsigargin described above.

At crayfish neuromuscular synapses, Ca^{2+} release from mitochondria is responsible for residual $[Ca^{2+}]_i$ after tetanus and for posttetanic potentiation. Tetraphenylphosphonium (TPP^+), an inhibitor of mitochondrial Ca^{2+} uptake and release (Aiuchi et al., 1985), blocked posttetanic potentiation but enhanced synaptic transmission during tetanus (Tang and Zucker, 1997). In *Drosophila* larvae, however, treatment with TPP^+ (5 μM for 10 min) did not affect FM1-43 loading into SV pools during tetanus (Figure 4D, column labeled 5 TPP^+). At 20 μM, TPP^+ decreased FM1-43 loading into boutons during tetanus and also reduced exocytosis of SVs (Figures 4D and 4G, columns labeled 20 TPP^+). Even at 20 μM, TPP^+ did not affect the FM1-43 loading into boutons after tetanus (Figure 4F, column labeled 20 TPP^+). These results suggest that mitochondrial Ca^{2+} is not contributing in the replenishment of the RP of this preparation.

Replenishment of SV Pools and Exocytosis during High K^+ or Veratridine Stimulation at Various Concentrations of External Ca^{2+}

To further clarify the roles of external and internal Ca^{2+} stores in the selective replenishment of SV pools, we examined FM1-43 loading into SV pools during stimulation with high K^+ or veratridine. When preparations were incubated in 90 mM K^+ plus FM1-43 in Ca^{2+}-free saline, no FM1-43 loading was observed in boutons (Figure 5A, row 1). However, when the same boutons were incubated in 90 mM K^+ plus FM1-43 in normal saline (2 mM Ca^{2+}), the periphery of boutons was stained with FM1-43 (Figure 5A, row 2) and completely destained after high K^+ unloading (Figure 5A, row 3). In contrast, when veratridine (10 μM), which opens voltage-gated Na^+ channels (Narahashi, 1974), was applied with FM1-43 in Ca^{2+}-free saline, the center of boutons was predominantly stained, and the majority of fluorescence remained after high K^+ unloading (Figures 5B1 and 5B2). When the same boutons were then incubated in veratridine plus FM1-43 in normal saline (2 mM Ca^{2+}), whole boutons were loaded with FM1-43 (Figure 5B3). The periphery was unloaded after subsequent high K^+ unloading, leaving FM1-43 in the center (Figure 5B4). These effects of veratridine were completely blocked by tetrodotoxin (6 μM) (data not shown).

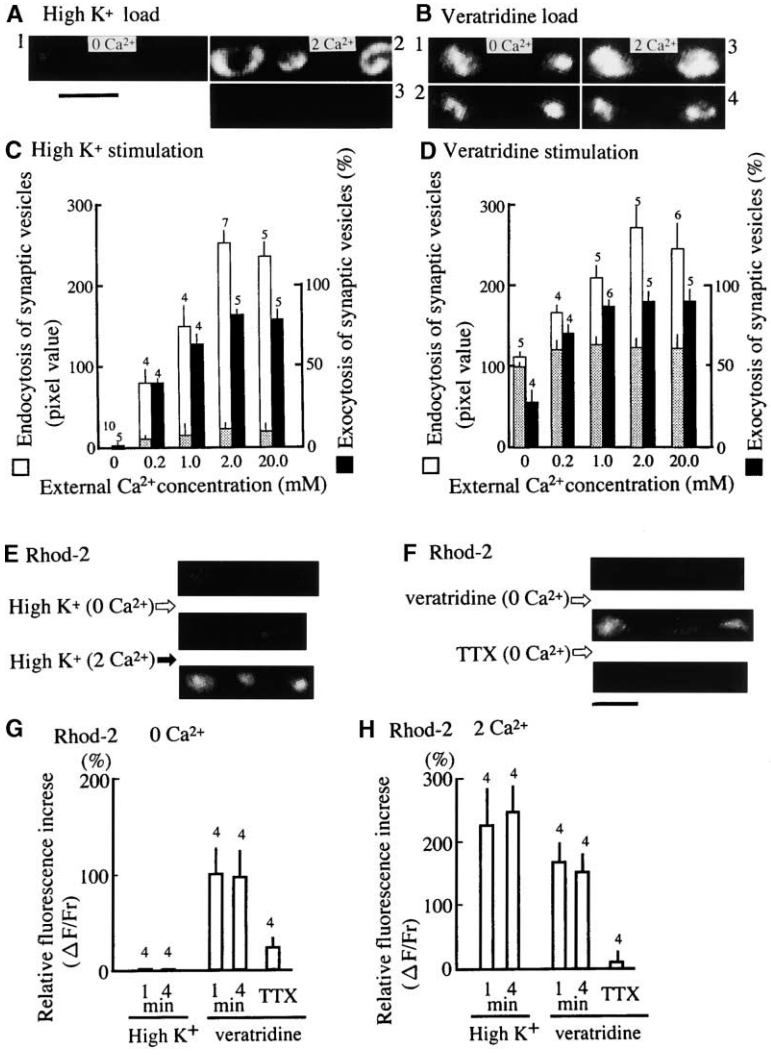

Figure 5. Endocytosis and Exocytosis of SVs and Changes in $[Ca^{2+}]_i$ during Stimulation with High K^+ or Veratridine

(A and B) FM1-43 fluorescence in boutons. In (A), the preparation was incubated for 5 min in Ca^{2+}-free saline containing 90 mM K^+ plus 10 μM FM1-43 and washed. No fluorescence was detected in the boutons (row 1). The same boutons were incubated for 5 min in normal saline (2 mM Ca^{2+}) containing 90 mM K^+ plus 10 μM FM1-43 and washed (row 2). Row 3 shows after high K^+ unloading. In (B), the preparation was incubated for 5 min in Ca^{2+}-free saline containing 10 μM veratridine plus 10 μM FM1-43 and washed (row 1). Row 2 shows after high K^+ unloading at 2 mM Ca^{2+}. The same boutons were incubated for 5 min in normal saline (2 mM Ca^{2+}) containing 10 μM veratridine plus 10 μM FM1-43 and washed (row 3). Row 4 shows after high K^+ unloading.

(C) Exocytosis and endocytosis of SVs during stimulation with 90 mM K^+ at various external Ca^{2+} concentrations. Boutons were preloaded with FM1-43 by high K^+ stimulation for 5 min. Exocytosis of SVs was determined by measuring the amount of FM1-43 released from boutons during stimulation with 90 K^+ for 2 min at various concentrations of Ca^{2+} (filled columns). Endocytosis of SVs was determined by measuring the FM1-43 taken up into boutons during stimulation with 90 mM K^+ for 2 min at various concentrations of Ca^{2+}. The FM1-43 fluorescence intensity in boutons before (height of whole columns at left) and after high K^+ unloading (height of stippled columns at left).

(D) Exocytosis and endocytosis of SVs during stimulation with veratridine at various external Ca^{2+} concentrations. Boutons were preloaded with FM1-43 by high K^+ stimulation for 5 min. Exocytosis of SVs was determined by measuring the amount of FM1-43 released from boutons during stimulation with 10 μM veratridine for 5 min at various concentrations of Ca^{2+} (filled columns). Endocytosis of SVs was determined by measuring FM1-43 taken up into boutons during stimulation with 10 μM veratridine for 5 min at various concentrations of external Ca^{2+}.

(E and F) Rhod-2 fluorescence in boutons. (E) depicts before (top) and 1 min after stimulation with 90 mM K^+ in Ca^{2+}-free saline (middle). The same boutons were then stimulated with 90 mM K^+ in normal saline (2 mM Ca^{2+}) (bottom). The image was taken 1 min after stimulation. (F) depicts before (top) and 1 min after stimulation with 10 μM veratridine in Ca^{2+}-free saline (middle). 6 μM tetrodotoxin (TTX) was added in the medium at 5 min after addition of veratridine (bottom). The image was taken 1 min after addition of TTX.

(G) Changes in the rhod-2 fluorescence intensity in boutons in Ca^{2+}-free saline. The resting fluorescence (Fr) was subtracted from the stimulation-induced value (Ft), and the result ($\Delta F = Ft - Fr$) was normalized to the resting fluorescence intensity (Fr) to give $\Delta F/Fr \times 100$. The intensity of rhod-2 fluorescence in boutons was measured at 1 min and 4 min after addition of 90 mM K^+ or 10 μM veratridine in Ca^{2+}-free saline and also measured 1 min after addition of 6 μM TTX in the presence of veratridine.

(H) Changes in the rhod-2 fluorescence intensity in boutons in normal saline (2 mM Ca^{2+}). For details, see figure legend Figure 5G.

Scale bar: 5 μm in (A), (B), (E), and (F).

Next, we examined the relationship between the external Ca^{2+} concentration and replenishment of SV pools during high K^+ or veratridine stimulation and also investigated the effects of external Ca^{2+} on exocytosis. Exocytosis and endocytosis of SVs induced by high K^+ stimulation diminished in parallel as the Ca^{2+} concentration decreased, and neither exocytosis nor endocytosis occurred at 0 Ca^{2+} (Figure 5C, the height of filled column and that of whole column on left in each set of columns). SVs endocytosed during high K^+ stimulation were predominantly incorporated into the ECP at all Ca^{2+} concentrations examined (Figure 5C, blank portion of left columns). Exocytosis and endocytosis of SVs induced by veratridine also reduced as the Ca^{2+} concentration decreased, but both still occurred at 0 Ca^{2+} (Figure 5D, filled columns and left whole columns). Incorporation of FM1-43 into the ECP during veratridine stimulation reduced as the Ca^{2+} concentration was decreased (Figure 5D, blank portion of left columns), while the uptake of FM1-43 into the RP did not change (Figure 5D, stippled portion of left columns). Thus, with both high K^+ and veratridine stimulation, the replenishment of ECP depended on the external Ca^{2+} concentration, while the endocytic replenishment of the RP did not.

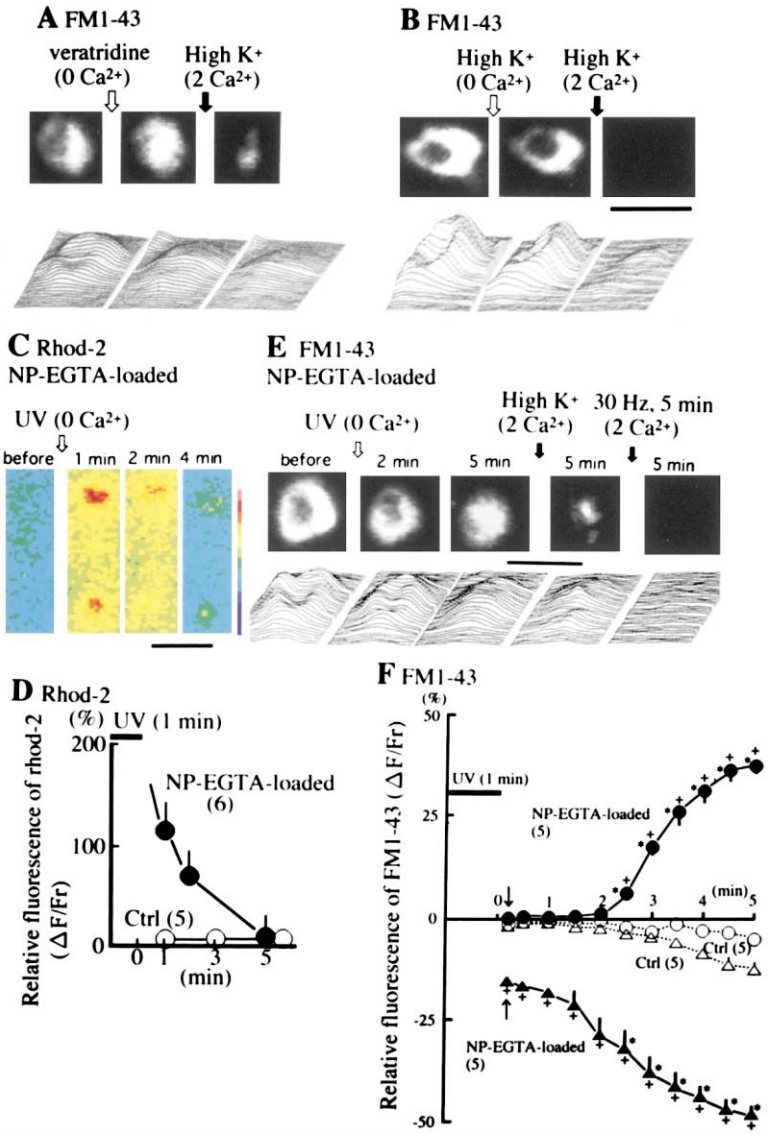

Figure 6. SV Translocation Induced by Veratridine or by Release of Ca^{2+} from a Caged Compound in Ca^{2+}-Free Saline

(A–D) Changes of FM1-43 fluorescence in boutons. Three-dimensional profiles (bottom) correspond to each fluorescence image (top). Boutons were first stained with FM1-43 by high K^+ loading. In (A), distributions of FM1-43 are shown before (left) and 5 min after treatment with 10 μM veratridine in Ca^{2+}-free saline (middle); after high K^+ unloading at 2 mM Ca^{2+} is shown (right). (B) depicts before (left) and 5 min after (middle) 90 mM K^+ treatment in Ca^{2+}-free saline; after high K^+ unloading at 2 mM Ca^{2+} is shown (right).

(C and D) Preparations were loaded with 10 μM NP-EGTA/AM and 10 μM rhod-2/AM. (C) depicts pseudo-colored rhod-2 images ($[Ca^{2+}]_i$). UV irradiation was applied for 1 min in Ca^{2+}-free saline at the arrow. (D) shows changes in the rhod-2 fluorescence intensity in whole bouton. NP-EGTA-loaded indicates preparations that had been loaded with NP-EGTA, and Ctrl indicates those without loading. Fr is the fluorescence intensity before UV irradiation and Ft at t after UV irradiation. $\Delta F = Ft - Fr$.

(E and F) Changes of the distribution of FM1-43 fluorescence in a bouton loaded with FM1-43 and NP-EGTA. In (E), fluorescence images before (first panel at left), 2 min after (second), and 5 min after (third) UV irradiation for 1 min in Ca^{2+}-free saline. The bouton was treated with high K^+ stimulation at 2 mM Ca^{2+} (fourth) and subsequently stimulated at 30 Hz for 5 min in normal saline (fifth). Three-dimensional profiles of FM1-43 distribution (bottom) correspond to each fluorescence image (top). In (F), time courses of FM1-43 fluorescence intensity in the peripheral (the area approximately 50% the size of radius from the edge of boutons) (triangles) and central (circles) regions of boutons. NP-EGTA-loaded (closed symbols) indicates preparations that are loaded with NP-EGTA and Ctrl (open symbols) indicates those without loading . Fr and Ft are the fluorescence intensity measured before and at t after UV irradiation and subtracted by the background value. $\Delta F = Ft - Fr$. Plus sign in (F) indicates significant difference ($p < 0.01$), compared with the value at the corresponding time in untreated controls. Asterisk indicates significant difference ($p < 0.01$), compared with the value at 15 s after UV irradiation (arrows).

Scale bar: 5 μm.

Changes in $[Ca^{2+}]_i$ in Boutons during High K^+ or Veratridine Stimulation in the Absence or Presence of External Ca^{2+}

To correlate changes in $[Ca^{2+}]_i$ with the selective replenishment of SV pools, we measured $[Ca^{2+}]_i$ in boutons with rhod-2/AM. In Ca^{2+}-free saline, the rhod-2 fluorescence intensity in boutons did not change during 90 mM K^+ stimulation (Figure 5E, middle; columns in Figure 5G labeled high K^+) but clearly increased during veratridine stimulation (Figure 5F, middle; columns in Figure 5G labeled veratridine). The increase in rhod-2 fluorescence induced by veratridine was inhibited by tetrodotoxin (Figure 5F, bottom; column in Figure 5G labeled veratridine and TTX). In normal saline (2 mM Ca^{2+}), the basal intensity of rhod-2 fluorescence in boutons increased to 221% ± 30% (nine preparations) of that in Ca^{2+}-free saline. The rhod-2 fluorescence intensity in boutons increased with 90 mM K^+ stimulation (Figure 5H, columns labeled high K^+) or veratridine (Figure 5H, columns labeled veratridine). Thus, high K^+ stimulation elevates $[Ca^{2+}]_i$ in boutons only in the presence of external Ca^{2+}, while veratridine increases $[Ca^{2+}]_i$ in boutons regardless of external Ca^{2+}. Since $[Ca^{2+}]_i$ elevation induced by veratridine in Ca^{2+}-free saline is probably due to release from internal stores, the above results suggest that Ca^{2+} release from internal stores underlies the replenishment of RP during veratridine stimulation.

Veratridine Translocates SVs from the Periphery to the Center of Boutons in the Absence of External Ca^{2+}

To replenish the RP, vesicular structures (endosomes, vesicles, or coated vesicles) have to move from the peripheral sites of endocytosis to the center. To examine

this translocation process, we first loaded FM1-43 into the periphery by high K^+ stimulation with 2 mM Ca^{2+} (Figures 6A and 6B, left). Subsequent treatment with veratridine (10 μM) in Ca^{2+}-free saline in the absence of FM1-43 redistributed a part of FM1-43 to the center of boutons (Figure 6A, middle). The result of this translocation became evident when subsequent high K^+ treatment at 2 mM Ca^{2+} destained the periphery (Figure 6A, right). When boutons loaded with FM1-43 by high K^+ stimulation were challenged again by high K^+ stimulation in Ca^{2+}-free saline, the fluorescence in the periphery was only slightly affected (Figure 6B, middle). The fluorescence completely disappeared after high K^+ unloading at 2 mM Ca^{2+} (Figure 6B, right), indicating the absence of translocation to the center. These results suggest that depolarization induced by veratridine per se does not translocate SVs to the center, but the elevation in $[Ca^{2+}]_i$ in the absence of external Ca^{2+} may initiate the translocation, as we showed above that veratridine increases $[Ca^{2+}]_i$ in the absence of external Ca^{2+}.

Ca^{2+} Release from a Caged Compound within Boutons Translocates SVs from the Periphery to the Center

To test our hypothesis, we elevated $[Ca^{2+}]_i$ in boutons using a caged Ca^{2+} compound. Preparations were loaded with NP-EGTA/AM (Ellis-Davies and Kaplan, 1994) and rhod-2/AM and then transferred to Ca^{2+}-free saline. UV irradiation for 1 min elevated $[Ca^{2+}]_i$ in these boutons (Figure 6C; closed circles in Figure 6D labeled NP-EGTA-loaded), but not in boutons loaded with only rhod-2 (open circles in Figure 6D labeled Ctrl). Thus, we confirmed that Ca^{2+} was released from the caged compound in boutons by UV irradiation.

We then examined the effect of Ca^{2+} uncaging on the FM1-43 distribution. Boutons, loaded with NP-EGTA/AM and stained with FM1-43 by high K^+ stimulation, were exposed to UV light in Ca^{2+}-free saline. The intensity of FM1-43 fluorescence measured in the periphery of boutons at 15 s after UV irradiation was about 84% of that before UV irradiation, while that in the center stayed at the same level (Figure 6F, the first closed triangle and circle indicated by arrows after UV irradiation). The FM1-43 fluorescence intensity in the periphery continuously declined, while that in the center increased with a delay of about 2 min (Figure 6E, the second and third panels; closed symbols in Figure 6F labeled NP-EGTA-loaded). The rapid changes in the fluorescence intensity after uncaging occurred with a similar time course in both peripheral and central regions, suggesting that vesicles translocated from the periphery to the center. The translocation of SVs to the RP was further confirmed by the observation that after high K^+ unloading with 2 mM Ca^{2+}, FM1-43 in the periphery disappeared, but that in the center remained (Figure 6E, fourth panel). The remained fluorescence in the center virtually disappeared after stimulation at 30 Hz for 5 min in normal saline (Figure 6E, far right), suggesting that SVs had translocated into the RP. In contrast, in the preparations without NP-EGTA loading, the distribution of FM1-43 fluorescence in boutons hardly changed (Figure 6F, open symbols labeled Ctrl). Thus, Ca^{2+} release from the caged compound in Ca^{2+}-free saline translocated SVs from the periphery to the center of boutons.

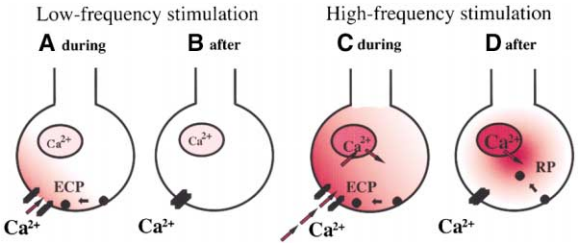

Figure 7. Hypothetical Representation of Relationship between Ca^{2+} Source and Endocytic Replenishment of SV Pools at Nerve Terminals of *Drosophila* Larvae

(A and C) Upon arrival of action potentials at the nerve terminals, voltage-gated Ca^{2+} channels open, and resulting Ca^{2+} influx elevates $[Ca^{2+}]_i$ in the vicinity of plasma membrane. In this situation, endocytosed SVs are predominantly incorporated in the ECP. During high-frequency tetanus, a continuous influx of Ca^{2+} increases $[Ca^{2+}]_i$ in the center of boutons. Ca^{2+} is taken up into and released from internal Ca^{2+} stores (C).

(B) $[Ca^{2+}]_i$ in boutons of the nerve terminals rapidly declines after low-frequency stimulation and endocytosis ceases.

(D) In contrast, after high-frequency stimulation, $[Ca^{2+}]_i$ remains elevated due to residual Ca^{2+} and Ca^{2+} release from internal stores. With this Ca^{2+} distribution, endocytosis continues and endocytosed vesicular structures are transported to the RP. Thus, two SV pools are replenished by endocytosed SVs through temporally distinct pathways, depending on the distribution of $[Ca^{2+}]_i$.

Thapsigargin blocks the uptake of Ca^{2+} into the internal stores, causing a high Ca^{2+} concentration in the center of boutons during the early period of tetanus and then depletion of internal Ca^{2+} stores in the late period. After tetanus, Ca^{2+} is not released from the depleted internal stores. In thapsigargin-treated boutons, endocytosed SVs are incorporated into the RP as well as into the ECP in the early period of tetanus, but incorporated only into the ECP in the late period, and no endocytosis occurs after tetanus (Figures 3 and 4).

Tetanic stimulation causes Ca^{2+} influx from the external solution and Ca^{2+} release from internal stores. In the low Ca^{2+} solution (0.2 and 0.4 mM Ca^{2+}), Ca^{2+} influx from external solution is significantly decreased, while Ca^{2+} release from internal stores is less affected. Thus, the relative concentration difference between the periphery and center is small. In this situation, SVs endocytosed during tetanus are incorporated into the RP as well as the ECP (Figure 2).

Discussion

Previously, we have shown that low-frequency nerve stimulation releases SVs only from the ECP, but with high-frequency tetanus, SVs in the RP are recruited for release in addition to SVs in the ECP (Kuromi and Kidokoro, 2000). In the present study, we demonstrated that two SV pools were selectively replenished by endocytosed SVs, depending on timing of endocytosis relative to tetanus and on the source of Ca^{2+}. Endocytosis of SVs after, as well as during, tetanus has been observed in retinal bipolar cells (von Gersdorff and Matthews, 1994), in hippocampal neurons (Ryan et al., 1996), and at frog neuromuscular junctions (Wu and Betz, 1996). However, the distinct characteristics of endocytosis that occurred during and after tetanus were not determined. In this study, we found at larval *Drosophila* neuromuscular synapses that, with normal external Ca^{2+}, SVs endocytosed during stimulation at both low- and high-frequency stimulation were predominantly loaded into the ECP, whereas SVs endocytosed after tetanus were mainly incorporated into the RP. Recently, it has been

demonstrated at frog neuromuscular junctions that FM2-10 loaded during tetanus is unloaded quickly, whereas the dye loaded after tetanus is unloaded following a delay, suggesting that FM2-10 is loaded into different SV pools, depending on the timing of endocytosis relative to tetanus (Richards et al., 2000). Their observation is consistent with our interpretation. In a *Drosophila* mutant, *shibire*, during recovery from paralysis at nonpermissive temperatures, two distinct pathways for endocytosis were revealed with an electron microscope. One pathway has a fast time course and emanates from the active zone, while the other pathway has a slower time course and occurs at sites away from the active zone (Koenig and Ikeda, 1996). These two pathways for endocytosis may correspond to our two types of endocytosis. The fast pathway may operate during nerve stimulation and be linked to the ECP, whereas the slow one may function after tetanus and be connected to the RP.

Extracellular Ca^{2+} is an important factor controlling endocytosis of SVs during stimulation (Ceccarelli et al., 1973; von Gersdorff and Matthews, 1994; Ramaswami et al., 1994; Sankaranarayanan and Ryan, 2001). The minimal external Ca^{2+} concentration that is required for induction of coated pits was determined to be 11 μM in lamprey synapses (Gad et al., 1998). Endocytosis of SV after tetanus was maintained even in Ca^{2+}-free saline in *Drosophila* boutons. Thus, compared with exocytosis, lower concentrations of external Ca^{2+} are sufficient for induction of endocytosis.

Based on the following findings, we conclude that the replenishment of ECP requires external Ca^{2+}. (1) During tetanic stimulation, FM1-43 was incorporated into the ECP only at the normal (2 mM) or higher concentrations of external Ca^{2+}, and the extent of FM1-43 uptake diminished in parallel with the decrease in external Ca^{2+} concentration. (2) With high K^+ depolarization, FM1-43 was taken up into boutons only in the presence of external Ca^{2+} and incorporated exclusively into the ECP. (3) During veratridine stimulation, FM1-43 was incorporated into the RP in the absence of external Ca^{2+} and, in parallel with external Ca^{2+} concentration, the extent of FM1-43 uptake into the ECP increased while that into the RP did not change.

We further conclude that the endocytic replenishment of RP does not require external Ca^{2+} but depends on Ca^{2+} release from internal stores, based on the following observations. (1) FM1-43 uptake into the RP after tetanus was not affected by removal of external Ca^{2+}. This result is consistent with the fact that FM1-43 uptake after tetanus was not affected by changing extracellular Ca^{2+} concentrations in hippocampal neurons (Ryan et al., 1996) and also compatible with the result that endocytosis of SVs during recovery from depletion at nonpermissive temperatures occurred in Ca^{2+}-free saline in *shibire* (Ramaswami, et al., 1994). (2) After depletion of Ca^{2+} from ER by thapsigargin, the uptake of FM1-43 into RP after tetanus was suppressed. (3) During a short tetanus in thapsigargin-treated boutons, FM1-43 uptake into the RP was not observed when internal Ca^{2+} stores had been depleted in Ca^{2+}-free saline. (4) Veratridine increased $[Ca^{2+}]_i$ in boutons in Ca^{2+}-free saline and caused incorporation of endocytosed SVs into the RP and translocation of SVs from the periphery to the center of bouton. (5) Finally, intracellular release of Ca^{2+} from the caged Ca^{2+} compound in Ca^{2+}-free saline translocated SVs from the periphery to the center of boutons. Thus, it appears that endocytosed SVs locate at the periphery of boutons where Ca^{2+} concentrations are high due to influx from the external source, whereas they are transported to the central region when Ca^{2+} is released intracellularly.

At present, it is unknown how endocytosed vesicles locate at or are transported to the regions of higher Ca^{2+} concentrations. Since this transportation was prevented by treatment with cytochalasin D (Kuromi and Kidokoro, 1998; Delgado et al., 2000), intact filamentous actin may play a role in transportation of SVs between two pools. ER vesicles are transported on actin filaments by myosin V (Tabb et al., 1998), and the complex of myosin V and vesicle proteins was disassembled in high Ca^{2+} concentrations in vitro (Prekeris and Terrian, 1997). SVs might be released at the region of high Ca^{2+} concentrations from actin filaments and accumulate there. Alternatively, phosphorylation of synapsin, due to activation of Ca^{2+}/calmodulin-dependent protein kinase, may release SVs from actin filaments (Hartwig et al.,1992; Greengard et al., 1993). A delay of about 2 min was found between the elevation of $[Ca^{2+}]_i$ and SV translocation after photolysis of the caged Ca^{2+} compound. This phenomenon suggests that SV translocation does not occur instantaneously after elevation of $[Ca^{2+}]_i$, but chemical and/or structural changes take place prior to the translocation of SVs. Figure 7 shows a hypothesis of endocytic replenishment of SV pools with special reference to Ca^{2+} source and Ca^{2+} distribution in the single bouton.

Experimental Procedures

Preparations

All experiments were carried out at synapses on muscles 6 and 7 of abdominal segment A-3 or A-4 in third instar larvae of a wild-type strain of *Drosophila melanogaster*, Canton S, as described previously (Kuromi and Kidokoro, 1998).

Optical Measurements

The optical measurement of FM1-43 fluorescence in boutons was performed as described previously (Kuromi and Kidokoro, 1998). Briefly, the preparations were viewed with an upright microscope (BX50WI; Olympus, Tokyo, Japan) equipped with DIC and epifluorescence optics. The preparations loaded with FM1-43 were excited with light of 488 nm (Polychrome II; TILL Photonics GmbH, Planegy, Germany), and light emitted at wavelengths above 530 nm was collected. Images were acquired with a CCD camera (C4880-81S, Hamamatsu Photonics, Hamamatsu, Japan) and processed with a Digital Celebris computer (GLST 5133, Nippon Digital Equipment, Tokyo, Japan) and an intracellular Ca^{2+} analysis system (Argus-HiSCA, Hamamatsu Photonics). For each preparation 10–15 brightly stained boutons with a diameter over 3 μm were selected by eye for quantification of the fluorescence intensity. After the background fluorescence at the surrounding region was subtracted, the mean pixel value was used as the FM1-43 fluorescence intensity in the bouton.

Confocal Microscopy

Confocal microscopy was carried out using an MRC-1024 (Bio-Rad, Hercules, CA) with an Argon ion laser mounted on an upright microscope (Axioplan 2, Zeiss). Living preparations stained with FM1-43 were viewed through fluorescein excitation and emission filters. Images were processed using the COMOS package (Bio-Rad).

$[Ca^{2+}]_i$ Measurement

The preparations were incubated for 40 min in nominally zero Ca^{2+} saline containing 10 μM rhod-2/AM and 0.03% pluronic F-127 in the dark at 22°C. The preparation was rinsed with HL3 medium three times. The fluorescence was excited at 545 nm and monitored at wavelengths above 580 nm. Rhod-2/AM stains mitochondria. However, mitochondria, stained with rhodamine-123 (20 μM for 20 min) (Johnson et al.,1980) were found distributed mainly in the periphery of boutons in this preparation (data not shown), while the fluorescence of rhod-2 was detected mainly in the center of boutons as shown in Figures 3, 5, and 6. We believe that rhod-2 fluorescence in the boutons reflects cytosolic Ca^{2+}.

Photolysis of Caged Ca^{2+}

For loading the caged Ca^{2+} compound, preparations were incubated for 40 min in nominally zero Ca^{2+} saline containing 10 μM nitrophenyl EGTA (NP-EGTA)/AM and 0.03% pluronic F-127. To monitor $[Ca^{2+}]_i$, 10 μM rhod-2/AM was also added in the above medium. When the effects of photolysis of caged Ca^{2+} compound on the SV distribution within single boutons were examined, boutons were stained with FM1-43 by high K^+ loading after they were loaded with NP-EGTA. Exposing the preparations to ultraviolet light (UV, 337 nm) for 1 min from the 75 W Xenon lamp (Plychrome II) performed photolysis of NP-EGTA. For control, the preparations were incubated for 40 min in nominally zero Ca^{2+} saline containing 0.03% pluronic F-127 and 1% DMSO.

Electrical Nerve Stimulation

To excite nerve terminals, pulses (1 ms and 2× threshold voltage) were delivered to the appropriate segmental nerve at 30 Hz via a regular glass suction electrode (20–30 μm inside diameter) or the ventral nerve cord via a large suction electrode (100–200 μm inside diameter) at 3 Hz.

Solutions

The composition of normal saline (Jan and Jan, 1976) was as follows: 130 mM NaCl, 36 mM sucrose, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM HEPES (pH 7.3). For Ca^{2+}-free saline, $CaCl_2$ was replaced with 2 mM $MgCl_2$, and 0.5 mM EGTA was added in normal saline. For nominally zero-Ca^{2+} saline, no EGTA was added to zero-Ca^{2+} saline. High K^+ saline contained 90 mM KCl in which NaCl was reduced by the equivalent amount. To examine the effects of external Ca^{2+} on the exocytosis or endocytosis, only the concentration of Ca^{2+} in normal saline was changed. HL3 medium (70 mM NaCl 115 mM sucrose, 5 mM KCl, 20 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, and 5 mM HEPES,) (Stewart et al., 1994) was used for measuring $[Ca^{2+}]_i$ to prevent vigorous muscle contraction.

Chemicals

FM1-43, pluronic F-127, and o-nitrophenyl EGTA/AM (NP-EGTA/AM) were from Molecular Probes (Eugene, OR); veratridine sulfate was from Sigma (St. Louis, MO); rhod-2/AM and tetraphenylphosphonium chloride (TPP^+) were from Dojindo (Kumamoto, Japan); thapsigargin and cyclopiazonic acid were from Alomone labs (Israel).

Statistical Analysis

Statistical analysis was performed by using Student's t-test.

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